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Original Article

The higher heparin-binding epidermal growth factor (HB-EGF) in missed abortion

Kemal Ozbilgin [a,*], Fatma Karaca [a], Afsin Turan [b], Can Köse [c], Seda Vatansever [a], Tayfun Ozcakir [d]

[a] Department of Histology and Embryology, School of Medicine, Celal Bayar University, 45050 Manisa, Turkey
[b] Department of Gynecology and Obstetrics, Ercis State Hospital, Van, 65400 Izmir, Turkey
[c] Department of IVF Unit, Tepecik State Hospital, 35170 Izmir, Turkey
[d] Department of Gynecology and Obstetrics, School of Medicine, Celal Bayar University, 45050 Manisa, Turkey

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ABSTRACT

Objective: Heparin-binding epidermal growth factor (HB-EGF) has pleiotropic biological functions in the female reproductive tract. HB-EGF has a function in the menstruation cycle, implantation, decidualization, placenta development, and also inhibition of apoptosis. This study aims to investigate a possible role of HB-EGF in missed abortion.
Materials and methods: Decidual and placental tissue samples were obtained from women with unwanted pregnancy as the control group and from women with missed abortions as the patient group. Immunohistochemistry was utilized to compare HB-EGF expression of fibroblast and decidual cells in uterine decidual stroma and fibroblasts and mesenchymal cells in placental villous stroma; the TUNEL technique was used to detect apoptotic cells within the decidual and placental tissues of the two groups.
Results: It was demonstrated that HB-EGF expression in both uterine decidual stroma and placenta stroma was increased in the missed abortion group (142.70 ± 12.80; 116.10 ± 14.16, respectively), compared with the normal pregnancy group (101.60 ± 14.18; 81.60 ± 10.74, respectively). It was also shown that there was no difference in TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labelling) positive cells between the uterine decidual stroma (11.4 ± 3%; 13.6 ± 3%, respectively), placental villous stroma (13.7 ± 3%; 15.9 ± 3%, respectively), and cytotrophoblast-syncytiotrophoblast cells (7.3 ± 2; 9.8 ± 3, respectively) of the two groups.
Conclusion: This data supports the hypothesis that increased HB-EGF expression in a missed abortion may prevent the discharge of the dead fetus.
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Introduction

Missed abortion, which is defined as a nonviable pregnancy that has been retained in the uterus without spontaneous passage for a number of weeks since the demise, implies that the uterus has "missed" recognizing the abnormal pregnancy [1]. Genetic and uterine abnormalities, endocrine and immunological dysfunctions, infectious agents, environmental pollutants, psychogenetic factors, and endometriosis are the most important known causes of missed abortion [2]. Although previous studies on missed abortion have mainly focused on diagnosis and treatment of it, we pay attention to the underlying causal factor.

Heparin-binding epidermal growth factor (HB-EGF) is a member of the EGF family and performs a variety of functions such as cell growth and differentiation in different cell systems [3]. Although HB-EGF is synthesized as transmembrane proteins (e.g., proHB-EGF), the secreted form (e.g., sHB-EGF) binds the cell receptors [4]. HB-EGF has two kinds of receptors which are tyrosine kinase HER/ErbB receptors and heparan sulfate proteoglycans (HSPG) [3]. HB-EGF stimulates HER1 and HER4 tyrosine kinase receptors but does not stimulate HER2 or HER3. HSPG is also necessary as a cofactor for HB-EGF to bind to its receptors, HER1 and HER4. HB-EGF induces receptor dimerization and autophosphorylation, leading to downstream signaling that affects an extensive array of pathways [5].

HB-EGF has been implicated as a participant in various normal physiological and pathologic processes such as cell attachments, chemotaxis, mitosis, and inhibition of cellular apoptosis. HB-EGF induces cell attachment through HER1 or HER4 [6], chemotaxis

* Corresponding author. Celal Bayar University School of Medicine, Department of Histology and Embryology, Uncubozkoy, Manisa, 45020, Turkey.
E-mail address: kemalozbilgin@yahoo.com (K. Ozbilgin).

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and mitosis of NIH 3T3 cells through HER1 signaling [7], and utilizes both MEK/ERK (Mitogen-activated protein kinase kinase/ extracellular signal-regulated kinases) and PI3K/Akt (Phosphoinositide 3-kinase/ Serine/threonine-specific protein kinase) pathways to inhibit cellular apoptosis [8].

HB-EGF also has a central role in the reproductive system of women during the menstruation cycle [9], implantation, and also decidualization [10]. HB-EGF mRNA is expressed throughout the menstrual cycle in humans [11] and is predominantly localized in the stromal compartment of the endometrium [12,13]. During the implantation process, HB-EGF is localized on the apical surface of human luminal epithelial cells and on the surface of pinopodes [14]. HB-EGF also plays an important role in the blastocyst adhesion and development [15,16].

HB-EGF plays a role in the decidualization of human endometrial stromal cells, induced via increased levels of prolactin and insulin-like growth factor binding protein-1 [17], and stimulates stromal cell growth via HER1 [12]. Decidualization *in vivo* occurs in response to steroid hormones, and hormonal control of gene expression in the endometrium is mediated by cAMP (Cyclic adenosine monophosphate) signaling [18]. In addition, Chobotova et al [17] reported that HB-EGF and its receptors (EGFR and ErbB4) have a function in decidualization of the human endometrium. It was reported that the local application of HB-EGF-soaked beads promotes decidualization in the mouse uterus [19].

HB-EGF is active during placental development and is present in the first trimester chorionic villi and decidua, in second trimester villi, and in term placenta [9]. Leach et al [20] reported that HB-EGF mRNA and protein have been expressed in villous and extravillous cytotrophoblast cells up to Week 35 of gestation in placentae from women who delivered preterm, but HB-EGF expression has been reduced about five-fold in preeclamptic pregnancies.

HB-EGF contributes significantly to the survival of trophoblastic cells during stress, induced by *in vitro* culture and can prevent apoptosis. The elimination of endogenous HB-EGF signaling during villous explants culture using the antagonist CRM197 significantly increased cell death among villous trophoblast cells [21]. The antiapoptotic function of HB-EGF is related to growth factors such as transforming growth factor (TGF)-β and tumor necrosis factor (TNF)-α, which have growth regulatory effects in both epithelial and stromal cells. TNFα and TGFβ significantly increase the levels of HB-EGF and implicate apoptosis in endometrial stromal cells [17].

Although HB-EGF and its receptors mediate the receptivity, maturation, and decidualization of endometrium, it facilitates the implantation and development of both placenta and embryo, but its function on missed abortion is not completely known. In this study, our aim is to determine the possible modulatory effects of HB-EGF on missed abortion and how to prevent the discharge of the dead fetus.

Materials and methods

Fifteen unwanted pregnancy (5–10 weeks gestational age) and 19 missed abortion (6–11 weeks gestational age) endometrial tissue samples were obtained with informed consent and in accordance with the requirements of the Ethics Committee of Celal Bayar University, Manisa, Turkey. The mean age of women was 27.53 years (range 21–37 years) for the normal pregnancy group and 28.74 years (range 18–41 years) for the missed abortion group.

Abortions were diagnosed by transvaginal ultrasound and were confirmed by repeat ultrasound prior to the dilation and curettage procedure. Chorionic villi and maternal decidua were separated and cleaned. Placental and decidual tissues were fixed in 10% buffered formalin solution and embedded in paraffin. The blocks were cut in 4–5 μm thick serial sections. The first sections of tissue were stained with HB-EGF primary antibody by means of an immunohistochemical technique and the second sections of tissue were stained by means of the TUNEL technique.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were used for immunohistochemical staining. The tissue samples were stored at 60°C overnight and were then dewaxed by xylene for 30 minutes. After dehydration of sections with ethanol, they were washed with distilled water. They were then treated with 2% trypsin (ab970, Abcam, Cambridge, UK) at 37°C for 15 minutes and incubated in 3% H_2O_2 solution for 15 minutes to inhibit endogenous peroxidase activity. Then, the sections were incubated with anti-Hb-EGF primary antibody (sc-1414; Santa Cruz, CA, USA) in a 1/100 dilution for 18 hours at +4°C. They were given three additional 5-minute washes in PBS (Phosphate buffered saline), followed by incubation with biotinylated immunoglobulin G and administration of streptavidin peroxidase (Histostain Plus kit Zymed 87-9999; Zymed, San Francisco, CA, USA). After washing the secondary antibody with PBS three times for 5 minutes, the sections were stained with DAB Substrate system containing diaminobenzidine (DAB, K007, DBS, Pleasanton, CA, USA) to detect the immunoreactivity, and then were stained with Mayer's hematoxylin (72804E, Microm, Walldorf, Germany) for counterstaining. They were covered with mounting medium (01730 Surgipath, Cambridge, UK) and observed with light microscopy (Olympus BX-40, Tokyo, Japan).

Immunohistochemical staining for HB-EGF was jointly scored on all cases using a semiquantitative system that is based on the H index [22]. Immunostaining intensity was categorized into the following scores: 0 (no staining), 1 (weak, but detectable staining), 2 (moderate staining), and 3 (intense staining). The H-score value was derived for each specimen by calculating the sum of the percentage of cells for fibroblast and decidual cells in uterine decidual stroma and fibroblasts and mesenchymal cells in placental villous stroma. Categorized by intensity of staining, multiplied by its respective score, by means of the formula:

$$\mathrm{H-score} = \sum \mathrm{Pi}(\mathrm{i}+1), \tag{1}$$

where i = intensity of staining with a value of 1, 2, or 3 (weak, moderate, or strong, respectively) and Pi is the percentage of stained epithelial cells for each intensity, varying from 0% to 100%. For each slide, five different fields were evaluated microscopically at 200 × magnification. H-score evaluation was performed by at least two investigators (K.O., S.V., F.K.) independently, blinded to the source of the samples as well as to each other's results and the average score was utilized.

TUNEL staining

We used the *in situ* apoptosis detection kit (DeadEnd Colorimetric TUNEL system, Millipore, S7100, USA & Canada) in order to detect apoptosis. Sections (5 μm) were cut from paraffin blocks of decidual and placental samples of the two groups. The sections were deparaffinized in xylene, rehydrated, and incubated with 20 μg/mL proteinase K for 10 minutes and rinsed in distilled water. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide. The sections were incubated with equilibration buffer for 10–15 seconds and TdT enzyme in a humidified atmosphere at 37°C for 60 minutes. They were put into pre-warmed working strength stop/wash buffer at room temperature for 10 minutes subsequently and incubated with anti-streptavidin–peroxidase for 45 minutes. The staining was done

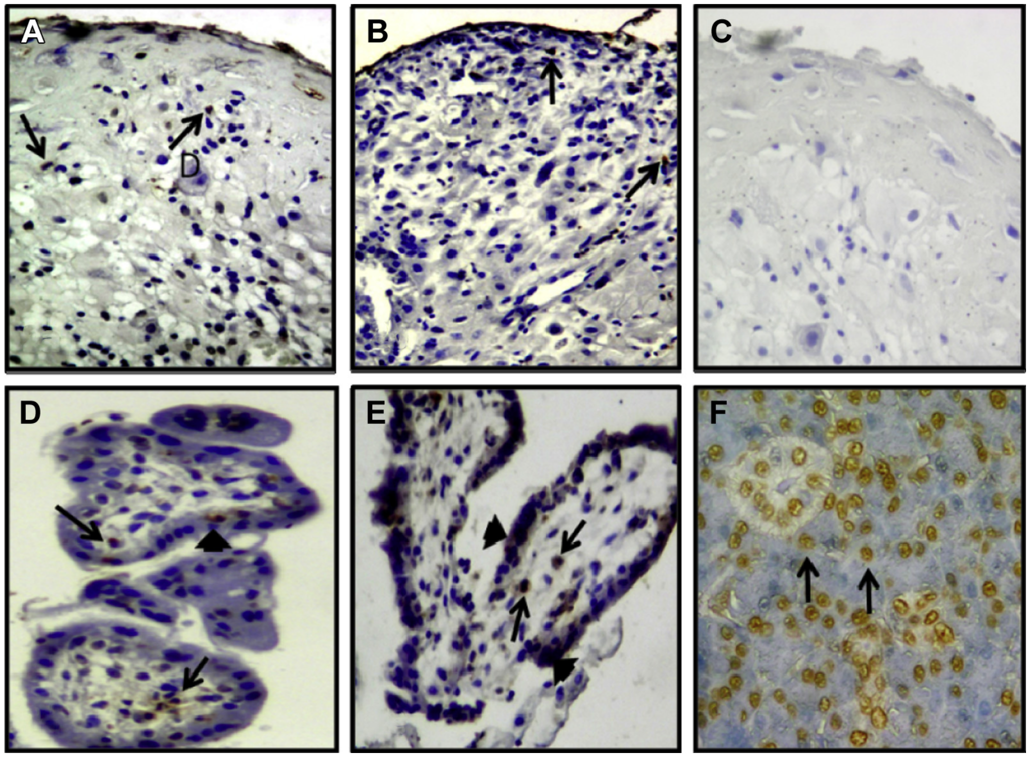

Fig. 1. The analysis of apoptosis in TUNEL labeling cells from normal pregnancy (A, D) and missed abortion group (B, E). A few apoptotic cells' nuclei (arrows) are stained with red and were seen in the decidua (A, B) and placenta (D, E). Stromal cells (arrows), syncytiotrophoblast-cytotrophoblast cells (arrowheads), stromal cells (arrows), negative control (C), and positive control (F) for TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labelling) (arrow) are also shown. Original magnification: A, B, D, E × 200; C, F × 400.

with DAB and nuclei were counterstained with Mayer's hematoxylin. As a negative staining control, TdT was omitted during the tailing reactions (Fig. 1C) and as a positive staining control have been used the lymphoid tissue section (according to the manufacturer's instructions; Fig. 1F). The apoptotic cells were counted from five areas in each section in a blinded manner, to determine the apoptotic rate. The relative incidence of apoptotic cells was calculated as:

$$(\%) = \text{number of TUNEL stained nuclei in a zone}/\text{number of hematoxylin stained nuclei in the same zone} \times 100. \quad (2)$$

Statistical analysis

Immunohistochemical values (mean ± SD) and the number of TUNEL positive cells data are summarized in Table 1. The comparison between the two groups was performed means of the Mann–Whitney *U* test. A *p* value <0.05 was considered significant. Statistical analysis was performed via SPSS statistical software, version 10.0 for Windows (SPSS Inc., Chicago, IL, USA). Error bars of H-score of HB-EGF expression and the number of TUNEL positive cells (%) are shown in Figs. 2 and 3, respectively.

Results

HB-EGF immunoreactivity was evaluated in both uterine decidual stroma and placental villous stroma of the normal pregnancy and the missed abortion groups. The H-scores of HB-EGF staining fibroblasts and decidual cells in the uterine decidual stroma were higher in the missed abortion group (142.70 ± 12.80) than the normal pregnancy group (101.6 ± 14.18; Table 1 and Figs. 2A, 4A, and 4B). The staining of fibroblasts and mesenchymal cells for HB-EGF was also lower in the placental villous stroma in the normal pregnancy group (81.6 ± 10.74) than in the missed abortion group (116.10 ± 14.16; Table 1 and Figs. 2B, 4C, and 4D).

We used the TUNEL assay to identify apoptotic cells by immunocytochemistry for both normal pregnancy and missed abortion groups. Values of TUNEL positive fibroblast and decidual cells in the

Table 1
H-score values of heparin-binding epidermal growth factor (HB-EGF) positive cells and number of TUNEL positive cells in missed abortion.

Group		HB-EGF uterine decidual stroma	HB-EGF placental villous stroma	TUNEL uterine decidual stroma	TUNEL placental villous stroma	TUNEL placenta cytotrophoblast-syncytiotrophoblast
Normal pregnancy	Mean ± SD	101.6 ± 14.18*	81.6 ± 10.74*	11.4 ± 3	13.7 ± 3	7.3 ± 2
	Median	100.50	85.00	10	1.3	7.5
Missed abortion	Mean ± SD	142.70 ± 12.80*	116.10 ± 14.16*	13.6 ±,3	15.9 ± 3	9.8 ± 3
	Median	145,50	116.00	14.5	0.165	8.5
Total	Mean ± SD	122.15 ± 24.84	98.85 ± 21.51	12.5 ± 3	148 ± 3	8.5 ± 3
	Median	121.00	95.50	12	15.5	8
	p	<0.0001	<0.0001	0.092	0.159	0.077

**p* < 0.01, Mann–Whitney *U* test.

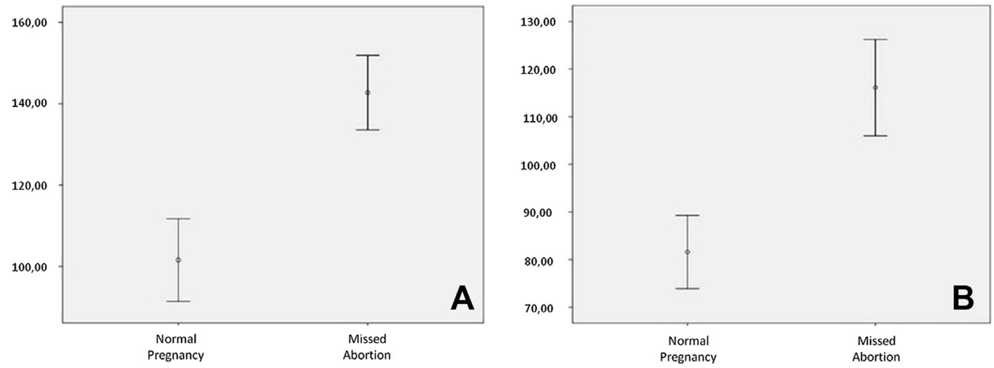

Fig. 2. Error bars of heparin-binding epidermal growth factor (HB-EGF) expression in uterine decidual stroma (A) and placental villous (B).

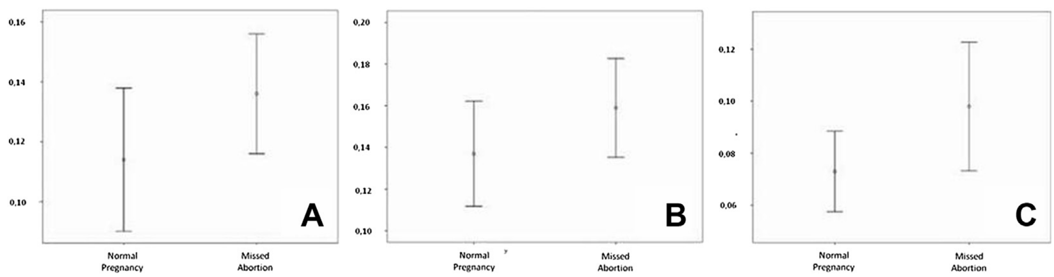

Fig. 3. Error bars of TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labelling) positive cells in uterine decidual stroma (A), placental villous stroma (B), and cytotrophoblast–syncytiotrophoblast cells (C).

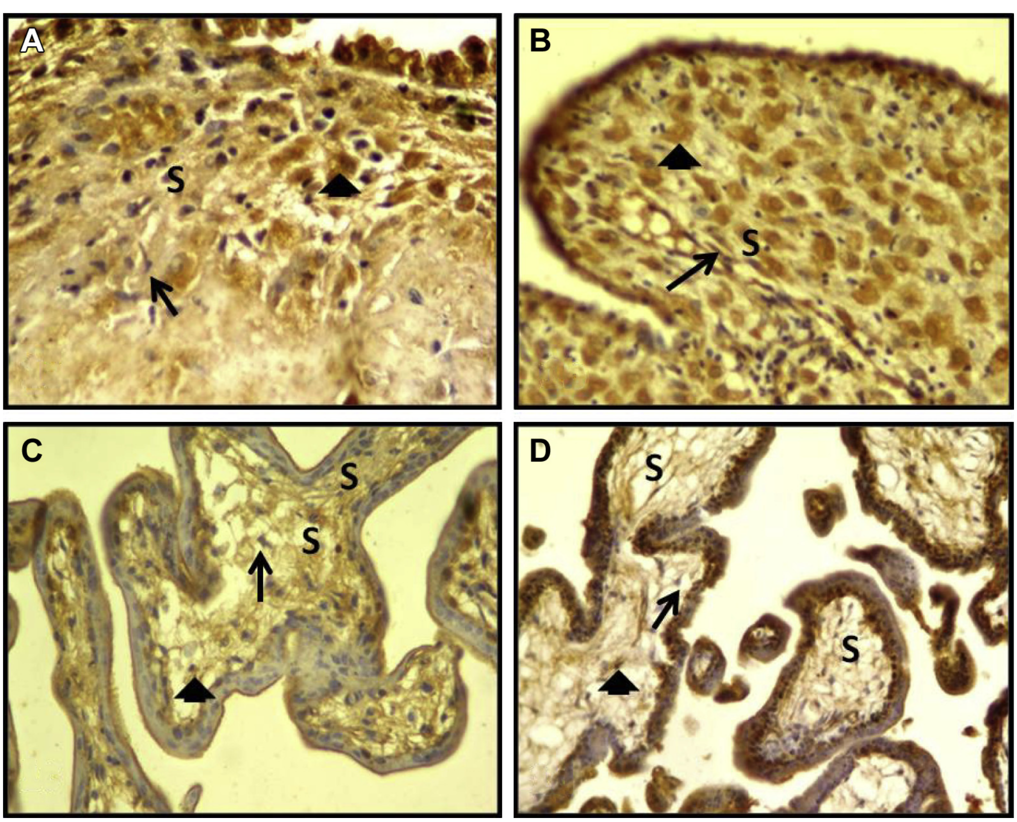

Fig. 4. The expression of heparin-binding epidermal growth factor (HB-EGF) in fibroblast (arrow) and decidual cells (arrowhead) in decidual stroma in normal pregnancy (A) and missed abortion group (B), and fibroblasts (arrow) and mesenchymal cells (arrowhead) in placental stroma in normal pregnancy (C) and in missed abortion group (D) by immunohistochemistry. The most intense immunoreactivities of HB-EGF were detected in the missed abortion group (B, D) rather than the normal pregnancy group (A, C). Original magnification × 400 (A, B), × 200 (C, D).

uterine decidual stroma were 11.4 ± 3% in the normal pregnancy group and 13.6 ± 3% the missed abortion group (Table 1 and Figs. 1A, 1D, and 3A). However, similar TUNEL positive cells of placental villous stromal cells were in the normal pregnancy (13.7 ± 3%) and missed abortion (15.9 ± 3%) groups (Table 1 and Figs. 1B, 1E, and 3B). In the placenta, TUNEL-positive syncytiotrophoblast-cytotrophoblast cells are 7.3 ± 2% in the normal pregnancy group (Fig. 1D) and 9.8 ± 3% in the missed abortion group (Table 1 and Figs. 1C, 1F, and 3C). There was no difference in TUNEL positive cell counts for both decidua and placenta cells between the two groups (Table 1 and Fig. 3).

Discussion

We demonstrated that there was a statistical significance in the immunohistochemical staining density of HB-EGF staining cells but there was no statistical significance of TUNEL positive cells between the normal fertile women (control) group and the missed abortion group. The current study claimed that in addition to the initiation of the pregnancy role, the HB-EGF may play crucial roles in the prevention of the discharge of the dead fetus.

HB-EGF performs two simultaneous functions during the human implantation as an attachment factor and a growth factor [23]. It was reported that HB-EGF plays an important role in the preparation of the uterine luminal epithelium for blastocyst attachment at the beginning of pregnancy [13]. HB-EGF, as a growth factor, accelerates the development of human embryos to the blastocyst stage and their subsequent hatching from the zona pellucida [24]. HB-EGF, as an attachment factor, upregulates many important proteins expressed from a uterine luminal epithelial surface such as integrin β3, leukemia inhibitory factor (LIF), and HOXA10 [25]. Integrin αvβ3 serves for osteopontin to mediate the embryo attachment [26]. LIF stimulates human embryo development to the blastocyst stage [27] and is required for embryo implantation [28]. HOXA10 was found in the human endometrium during the mid-secretory phase abundantly and may be involved in implantation and decidualization of endometrium during early pregnancy [29]. HOXA10 induction by P4 during the window of implantation leads to a blockage in the stromal cell cycle, facilitating decidualization [30].

Previous studies reported that HB-EGF plays a role in the decidualization of human endometrial stromal cells [31]. Our immunohistochemical results showed HB-EGF expression was found on the decidual cells of both groups, but they were more intense in the missed abortion group (Figs. 3A, 4A, and 4B). Chobotova et al [17] reported that endometrial stromal cells are treated with 8-Br-cAMP, which is known to artificially induce decidualization; the soluble form of HB-EGF is upregulated. It was reported that two different inhibitors of HB-EGF activity, the diphtheria toxin analogue CRM197 and neutralizing HB-EGF antibodies, result in decreased levels of prolactin and insulin-like growth factor-binding protein-1. It was suggested that HB-EGF induces decidualization through the increase of stromal cell production of prolactin and insulin-like growth factor binding protein-1 [17]. In addition, Karpovich et al [32] reported that HB-EGF could result from the upregulation of interleukin (IL)-11 secretion, which plays a role in the decidualization process in mice. However, female mice without the IL-11Rα gene are infertile due to defective differentiation of the stroma in response to an implanting blastocyst, leading to the resorption of the embryo [33].

A successful pregnancy in humans depends on the deep invasion of the maternal decidua by extravillous trophoblast cells (EVTs), a process regulated by autocrine and paracrine signals in the decidual-trophoblast microenvironment. Gonzalez et al [34] reported that decidual cells are the biosensors for embryo quality and placed HB-EGF and IL-1b among the critical factors indicative of the selective response. We found that HB-EGF expression in the uterine decidual stromal areas is higher in the missed abortion group than in the fertile group (Figs. 3A, 4A, and 4B) and suggest that HB-EGF expression might support the stroma integrity, thus preventing the discharge of the dead fetus. HB-EGF may have a mitogenic function in the human endometrial cells, as well as an invasion function in trophoblastic cells. HB-EGF induces mitosis for fibroblasts and smooth muscle cells and contributes to wound healing [3,35] for endometrial stromal cells and many other cell types [12]. HB-EGF is also considered to be important in trophoblast invasion and placental development leading to enhanced cell migration [36]. When supplementing co-cultures of undifferentiated ESC and trophoblast spheroids with HB-EGF, undifferentiated ESC provides a matrix that, in conjunction with HB-EGF, becomes optimized to support trophoblast spreading [3].

HB-EGF expression was observed to exist both in uterine decidual stroma and in placental villous stroma of the two groups (Figs. 3A, 3B, 4A, and 4D), which indicates that HB-EGF is a survival factor during the establishment of pregnancy and placental development [17]. Birdsall et al [9] reported that HB-EGF is abundant in placental tissue during all three trimesters. The extensive expression of HB-EGF in trophoblasts could be vital for their invasive activities during pregnancy [20]. It is known that the paucity of HB-EGF in placenta leads to preeclampsia [37]. However, an addition of HB-EGF to term villous explants cultured at 2% O_2 inhibits trophoblast apoptosis, demonstrating its persistent role as a survival factor [21]. These findings highlighted the importance of HB-EGF in success of early placentation and in protecting trophoblast cells from the damaging effects of hypoxia encountered throughout gestation.

HB-EGF protects from apoptosis and is accepted as a survival factor [17]. In this study, we observed very rare TUNEL positive cells in the normal pregnancy group (Fig. 2A and D) and in the missed abortion group (Fig. 2B and E). There was no significant difference in TUNEL positive cells between two groups, which suggests that minimal apoptosis may maintain pregnancy and prevent from abortion. By contrast, the increased expression of FasL in decidual lymphocytes, the expression of Fas in EVT and the apoptosis of interstitial EVT *in situ* have been reported in spontaneous abortion of humans [38]. It is known that the dysregulation of HB-EGF and the deficiencies in trophoblast function could lead to preeclampsia. Although it remains to be determined during the course of gestation, HB-EGF levels are found to be reduced in preeclamptic pregnancies for the first time. Trophoblast invasion is shallow in preeclampsia, possibly due to the lack of HB-EGF-induced cell migration and the rise in apoptosis, exacerbated by reduced cytoprotection. Patients delivering preterm without hypertensive disorder produce placentae with normal levels of HB-EGF [20].

It is known that placentation is initiated in a low oxygen environment and hypoxia leads to apoptotic death. By means of specific antagonists of HB-EGF signaling, it can be demonstrated that HB-EGF inhibits apoptosis due to hypoxia. Hung et al [39] demonstrated that reactive oxygen species are generated *in vitro* in a reoxygenation injury model, leading to apoptosis of the trophoblasts [40]. HB-EGF can also prevent apoptosis induced by reoxygenation injury in HTR-8/SVneo cells by signaling through its receptors, HER1 and HER4 [41]. Although HB-EGF is downregulated within 30 minutes of elevating the O_2 concentration from 2% to 20%, accumulated sHB-EGF generated by trophoblasts at low oxygen may protect against a sudden exposure to oxygenated maternal blood in the uterine environment. By contrast, only HB-EGF inhibits apoptosis in cytotrophoblasts cultured at low oxygen [42]. It is proposed that insults such as reoxygenation injury are not always tolerated and can lead to placental pathologies [43]. The activity of the EGF signaling system could be a critical variable that keeps oxidative stress in check.

Previous studies reported that decreased levels of HB-EGF leads to pregnancy complications such as preeclampsia and small gestational age, due to aberrant trophoblast invasion [44] and also to intrauterine growth restriction due to elevated apoptosis [45]. Current data demonstrated that high HB-EGF expression is found in two important tissue types (decidual and trophoblastic cells), and TUNEL positive cells are very low in both groups. In light of these observations, HB-EGF acts as a decidualization factor, a trophoblastic invasion modulator, and a potent cytoprotective agent in both groups.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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